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Commentary

Bridging the gap between protein carboxyl methylation and phospholipid methylation to understand glucose-stimulated insulin secretion from the pancreatic β cell

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ABSTRACT

Recent findings have implicated post-translational modifications at C-terminal cysteines [e.g., methylation] of specific proteins [e.g., G-proteins] in glucose-stimulated insulin secretion [GSIS]. Furthermore, methylation at the C-terminal leucine of the catalytic subunit of protein phosphatase 2A [PP2Ac] has also been shown to be relevant for GSIS. In addition to these two classes of protein methyl transferases, a novel class of glucose-activated phospholipid methyl transferases have also been identified in the β cell. These enzymes catalyze three successive methylations of phosphatidylethanolamine to yield phosphatidylcholine. The ''newly formed'' phosphatidylcholine is felt to induce alterations in the membrane fluidity, which might favor vesicular fusion with the plasma membrane for the exocytosis of insulin. The objectives of this commentary are to: (i) review the existing evidence on the regulation, by glucose and other insulin secretagogues, of post-translational carboxylmethylation [CML] of specific proteins in the β cell; (ii) discuss the experimental evidence, which implicates regulation, by glucose and other insulin secretagogues, of phosphatidylethanolamine methylation in the islet β cell; (iii) propose a model for potential cross-talk between the protein and lipid methylation pathways in the regulation of GSIS and (iv) highlight potential avenues for future research, including the development of specific pharmacological inhibitors to further decipher regulatory roles for these methylation reactions in islet β cell function.

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1. Introduction

Glucose-stimulated insulin secretion [GSIS] from pancreatic β cells is largely mediated via generation of soluble second messengers such as cyclic nucleotides, hydrolytic products of phospholipases [A_2, C and D] and adenine nucleotides [1,2]. While, the exact molecular and cellular mechanisms underlying glucose-induced insulin release remain only partially understood, it is widely accepted that, following its entry into the β cell, glucose is metabolized with a resultant increase

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Abbreviations: CML, carboxylmethylation; GSIS, glucose-stimulated insulin secretion; MTA, methylthiodeoxyadenosine; PP2A, protein phosphatase 2A; PP2Ac, catalytic subunit of PP2A; SAM, S-adenosylmethionine

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in the ATP/ADP ratio. Such an increase in the ATP levels culminates in the closure of ATP-sensitive potassium channels localized on the plasma membrane which results in membrane depolarization followed by influx of extracellular calcium through the voltage-sensitive calcium channels. An increase in intracellular calcium has been shown to be critical for the transport of insulin-containing secretory granules to the plasma membrane for fusion and release of insulin. Several lines of evidence clearly implicate post-translational modifications of proteins in the stimulus-secretion coupling of events leading to GSIS. These include, but are not limited to, phosphorylation/dephosphorylation, isoprenylation, carboxylmethylation [CML], fatty acylation, etc. [1–5]. In addition, extant data also support the formulation that modification of endogenous lipids [e.g., methylation of phosphatidyl-ethanolamine] may also play critical roles in β cell activation and insulin secretion induced by glucose and other insulin secretagogues.

It is well established that the methylation reactions, including the protein and lipid methylation involve transfer of methyl groups from a methyl donor, such as the S-adenosyl methione [SAM] to the respective methyl acceptors [e.g., proteins and lipids]. SAM, which was first discovered in 1952, is synthesized endogenously from ATP and methionine by methionine adenosyl-transferase [6–9]. Individual steps involved in the production, consumption and regeneration of SAM are shown in Fig. 1. In the first step of this cycle, the SAM-dependent methyl transferases [e.g., protein or lipid methyltransferases] utilize SAM as a methyl donor substrate to produce S-adenosyl homocysteine as a byproduct. This, in turn, is hydrolyzed to homocysteine and adenosine by the enzyme S-adenosylhomocysteine-hydrolase. The homocysteine is then recycled back to methionine involving the transfer of a methyl group from 5-methyl-tetrahydrofolate, by the enzyme methionine synthase. This methionine is converted back to SAM thus completing the cycle. The precise mechanisms underlying the regulation, by glucose or its intermediates, of the SAM cycle in the islet β cell have not been addressed adequately yet. Nonetheless, the potential utility of SAM as a methyl donor for the islet endogenous protein and lipid methylation has been well described [6–9]. Such protocols involved either labeling of intact β cells with [^{3}H]methionine to label the endogenous SAM pools (Fig. 1) or utilizing the [^{3}H]SAM in vitro experiments involving total cell lysates as well as isolated subcellular fractions [e.g., plasma membrane and secretory granules].

Using these experimental approaches, several earlier studies have quantitated protein and lipid methylation in the pancreatic β cells [2,4,5 and see above]. However, potential cross-talk between these two signaling pathways in the sequence of events leading to fusion of secretory granules with the plasma membrane, and the subsequent release of insulin has not been addressed before. Please note that the majority of the work on the regulatory aspects of phospholipid methylation in GSIS were reported during mid to late 1980s and research findings on the CML of small molecular mass as well as trimeric G-proteins in the islet β cell were published during the 1990s. Further, regulation of protein phosphatase function by CML became evident during late 1990s. Therefore, it is my hope that this commentary will generate debate and interest among the islet researchers to begin to further evaluate the regulatory roles for SAM as a methyl donor for various methylation steps in the islet β cell leading to physiological insulin secretion. Second, I am also hopeful that this article will provide the basis for future investigations to understand regulatory aspects of protein and lipid methylation steps in glucose-mediated trafficking of insulin-laded secretory granules toward the plasma membrane for fusion and release of insulin.

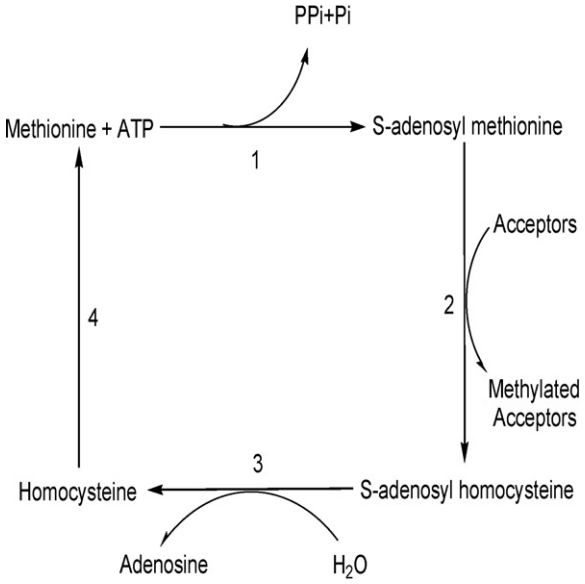

Fig. 1 – Individual steps in SAM cycle: individual steps involved in the production, consumption and regeneration of SAM are represented in this figure. Enzymes involved are: 1, SAM synthase; 2, SAM-dependent demethylases, which include protein and phospholipid methylating enzymes; 3, S-adenosyl homocysteine hydrolase and 4, methionine synthase.

Therefore, the principal objectives of this commentary are: (i) to provide a brief overview of the existing evidence on glucose-mediated regulation protein [specifically, the CML] and lipid methylation in the islet β cell and (ii) to propose a model for potential cross-talk between these two signaling pathways in the regulation of GSIS. In the last section of this commentary, I will attempt to highlight potential avenues for future research, including the development of specific pharmacological inhibitors to further decipher regulatory roles for these methylation reactions in islet β cell function.

2. Studies on protein carboxyl methylation in islet β cells

2.1. *Evidence for a requisite role for the methylation of the C-terminal cysteines of specific G-proteins in GSIS*

In addition to the regulation of GSIS by adenine nucleotides [see above], previous studies have examined the possible contributory roles for guanine nucleotides [i.e., guanosine triphosphate; GTP] in physiologic insulin release. For example,

using selective inhibitors of GTP biosynthetic pathway [e.g., mycophenolic acid], a permissive role for GTP in GSIS was first established in [10–12]. Although the exact molecular and cellular mechanisms underlying the regulatory role[s] of GTP remain only partially understood, available evidence indicates that it might involve activation of one [or more] G-proteins. Two major classes of G-proteins have been identified in β cells. The first group consists of trimeric G-proteins comprised of αβγ subunits. These G-proteins are involved in the coupling of various receptors to their intracellular effectors, such as adenylate cyclase, phosphodiesterase or several phospholipases [13,14]. The second group of G-proteins is comprised of low molecular mass, monomeric G-proteins, which are involved in sorting of proteins as well as trafficking of secretory vesicles [15].

It has been known for a long time that both monomeric G-proteins as well as the γ subunits of trimeric G-proteins undergo post-translational modifications, such as isoprenylation and methylation at their C-terminal cysteine residues [often referred to as the CAAX motif; Fig. 2]. The first of a four-step modification sequence includes incorporation of a 15-carbon [farnesyl] or 20-carbon [geranylgeranyl] isoprenoid moiety, which is derived from mevalonic acid, onto a cysteine residue on the carboxyl terminus of the candidate G-proteins. This is followed by the proteolytic cleavage of several amino acids [up to a maximum of three]. Often a CML step then modifies the newly exposed carboxylate anion of the cysteine. In some cases [not depicted in Fig. 2], the covalent addition of a long-chain fatty acid, typically palmitate, completes the cascade. This is felt to render the modified G-proteins more hydrophobic and able to associate tightly with membranes [their putative site of action] for interaction with their respective effectors. Since the isoprenylation of G-proteins occurs shortly after their synthesis, and because ''half-lives'' of prenylated proteins are rather long, this is not likely to be an acute regulatory step; however in many cases, prenylation is necessary to allow G-proteins to intercalate into the relevant membrane compartment. In contrast, the CML step is subject to acute regulation at the level of the ''on'' step [addition of methyl groups] and/or the ''off'' step [removal of methyl groups]. Earlier studies have demonstrated relevance of post-translational modifications of these proteins in physiological insulin secretion [2,4,5,16–19]. Of contextual importance to this current commentary are potential contributory roles for the protein CML steps in GSIS; these aspects are discussed below.

As mentioned above, available data in the islet β cell clearly suggest that the carboxylmethylated of prenylated cysteine is acutely regulated under conditions of GSIS. Using normal rat islets and clonal β cell preparations, we have been able to demonstrate transient stimulation, by glucose, of the CML of small G-proteins [e.g., Cdc42 or Rap1] and the γ subunits of trimeric G-proteins [19]. Further, we have demonstrated that these proteins are rapidly demethylated in isolated β cell preparations [17]. We have also characterized protein prenyl methyl transferase activity in the islet β cell [20]. The carboxyl methyltransferase catalyzes the incorporation of a methyl group onto the carboxylate anion of the prenylated cysteine via an ester linkage (Fig. 2). It utilizes the intracellular SAM as the methyl donor. In addition, a methylesterase activity that specifically cleaves the methylated residue has also been identified in the islet β cell [17]. Previously, we have identified

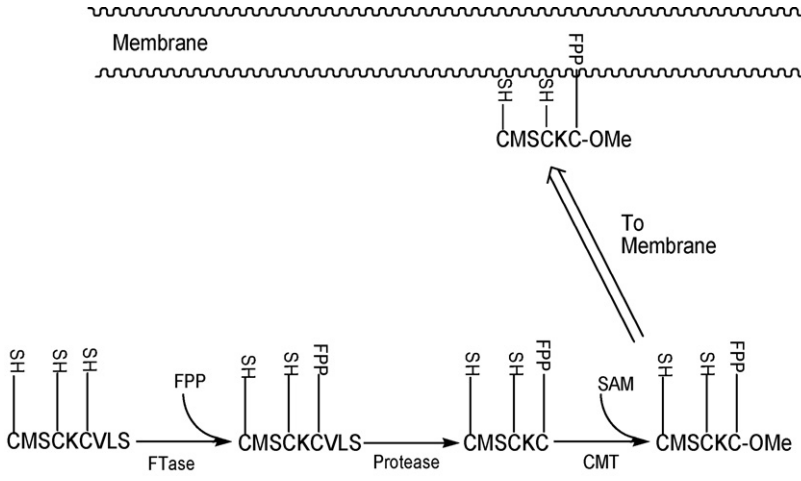

Fig. 2 – Post-translational prenylation and CML of small G-proteins: the first of the three-step reaction is incorporation of either a 15 [farnesyl]- or a 20 [geranylgeranyl]-carbon derivative of mevalonic acid into the COOH-terminal cysteine via a thioether linkage. This reaction is catalyzed by either the farnesyl or geranylgeranyl transferases, respectively. After this, the three amino acids after the prenylated cysteine are removed by a protease of microsomal origin, thereby exposing the carboxylate anion. This site is then methylated by a carboxyl methyl transferase, which transfers a methyl group onto the carboxylate group using SAM as the methyl donor. We have shown that the CML of specific G-proteins [e.g., Cdc42] increases their hydrophobicity and translocation to the membrane fraction. Please note that, in addition to these, certain G-proteins [e.g., H-Ras] have also been shown to undergo palmitoylation at a cysteine residue, which is upstream to the prenylated cysteine. It is thought that palmitoylation provides a ''firm'' anchoring for the modified protein into the cell membrane for optimal interaction with its respective effector proteins. FPP, Farnesyl pyrophosphate; FTase, farnesyl transferase; CMT, carboxyl methyl transferase.

several carboxylmethylated proteins in the pancreatic β cell. These include Cdc42, Rap1, Rac1, H-Ras, the γ subunits of trimeric G-proteins as well as the nuclear lamin-B [20,21]. Earlier findings from our laboratory have also demonstrated that glucose transiently increases the CML and membrane association of Cdc42 thereby facilitating its interaction and activation of phospholipase C with a concomitant increase in the secretion of insulin [19]. Moreover, acetyl farnesyl cysteine, a cell-permeable specific inhibitor of the CML of prenylated cysteines, markedly attenuated glucose-stimulated CML of Cdc42, phospholipase C activation and insulin secretion [19]. Our hypothesis that such a post-translational modification of Cdc42 is required for insulin release was further strengthened by our findings, which suggested that acetyl geranyl cysteine, an inactive analog of acetyl farnesyl cysteine, failed to inhibit glucose-mediated effects on the CML of Cdc42, phospholipase C activation and insulin secretion [19]. Around same time, independent studies from Leiser et al. [5] have also utilized these specific probes to determine the relative contribution of Rap1, another monomeric G-protein, in glucose- and calcium-mediated insulin secretion.

Follow-up studies from our laboratory have utilized similar experimental approaches and probes to decipher the roles of the CML of Gγ subunits in GSIS [17]. The γ subunits of trimeric G-proteins [γ_1, γ_2, γ_5 and γ_7 isoforms] were found to be carboxylmethylated in normal rat islets, human islets and clonal β cell preparations. Of these, GTPγS, a non-hydrolyzable analog of GTP, selectively stimulated the CML of γ_2 and γ_5 isoforms. Exposure of intact β cells to either of two receptor-independent agonists, namely a stimulatory concentration of glucose or a membrane depolarizing concentration of KCl resulted in a rapid [within 30 s] and sustained [at least up to 60 min] stimulation of the CML of γ subunits. Mastoparan, a tetradecapeptide from wasp venom, which directly activates G-proteins and insulin secretion from β cells, also stimulated the CML of γ subunits in intact insulin-secreting cells. Stimulatory effects of glucose or KCl were not demonstrable after removal of extracellular calcium [using EGTA] or depletion of intracellular GTP [using mycophenolic acid], implying regulatory roles for calcium fluxes and GTP. However, the methyl transferase itself was not directly activated by either calcium or GTP. The stimulatory effects of mastoparan were resistant to removal of extracellular calcium, implying a mechanism of action that is different from glucose [via its metabolism] or KCl [via membrane depolarization and calcium influx] but also suggesting that dissociation of the αβγ trimer is conducive to the CML of γ subunits. Indeed, pertussis toxin, which inactivates the inhibitory class of trimeric G-proteins [e.g., G_i or G_o] via ADP-ribosylation of their respective α subunits, also markedly attenuated the stimulatory effects of glucose, KCl or mastoparan without altering the rise in intracellular calcium induced by glucose or KCl. Glucose-induced CML of γ_2 and γ_5 isoforms was vitiated by coprovision of any of three structurally different cyclooxygenase inhibitors [e.g., aspirin, ibuprofen and indomethacin [17]]. Conversely, exogenous prostaglandin E_2, which activates G_i and G_o in β cells and which thereby would dissociate the α subunit from βγ, stimulated the CML of γ_2 and γ_5 isoforms and reversed the inhibition of glucose-stimulated CML of γ subunits elicited by cyclooxygenase inhibitors. These data indicate that γ subunits of trimeric G-proteins undergo a glucose- and calcium-regulated methylation–demethylation cycle in insulin-secreting cells, findings that may imply an important role in β cell function. To the best of my knowledge, these findings represented the first example of the regulation of the post-translational modification of G-protein γ subunits via non-receptor-mediated activation mechanisms, which are apparently dependent on calcium influx and the consequent activation of phospholipases releasing arachidonic acid.

In addition to demonstrating potential regulatory roles for CML of islet endogenous small molecular weight G-proteins and the γ subunits of trimeric G-proteins in GSIS, previous studies from our laboratory have also characterized the prenyl cysteine methyltransferase activity in insulin-secreting cells and normal rat islets [20]. Such an activity was monitored by the methylation of an artificial substrate [e.g., acetyl farnesyl cysteine] with [^{3}H]SAM as methyl donor. Subcellular fractionation studies revealed that the prenyl cysteine methylating enzyme is predominantly localized in the plasma membrane fraction as well as the endoplasmic reticulum. Furthermore, we observed that exogenous GTP had no demonstrable effect on the ability of the methyl transferase to methylate acetyl fanesyl cysteine suggesting that such an activity may be constitutively active within the β cell, and that the methylation of target G-proteins in an intact β cell is regulated by the access of these G-proteins to the methyltransferase, as well as their active GTP-bound conformation [20]. The reader is referred to a recent review [21] for a summary of existing body of evidence on protein C-terminal cysteine methylation reactions in insulin-secreting cells and their relevance to physiologic insulin secretion.

2.2. *Methylation of C-terminal leucine of the catalytic subunit of protein phosphatase 2A [PP2Ac]*

Unlike the small G-proteins or the γ subunits of trimeric G-proteins, the PP2Ac undergoes reversible methylation at its C-terminal leucine^{309} residue [22,23] (Fig. 3). PP2Ac has been shown to be highly conserved, with >70% sequence homology among different species [24]. The six C-terminal amino acid residues (TPDYFL) are absolutely conserved in all known PP2Ac subunits. The structure of protein phosphatase methyltransferase-1 has been elucidated recently [25]. Previous studies from our laboratory examined putative regulatory roles for the CML of PP2Ac in insulin secretion elicited by stimulatory concentrations of glucose. In this context, we reported the CML of a predominantly cytosolic 36-kDa protein in intact normal rat islets and INS-1 cells [23]. The CML of the 36 kDa protein was resistant to inhibition by acetyl farnesyl cysteine a competitive substrate for cysteine methyl transferases [see above], suggesting that the methylated C-terminal amino acid is not cysteine. The methylated protein was identified as PP2Ac by immunoblotting. Okadaic acid, but not 1-nor-okadaone, its inactive analog, inhibited the CML of PP2Ac and PP2A activity in the cytosolic fraction derived from normal rat islets and clonal β cells.

The next question is how important and/or what are the contributory roles for the CML of PP2Ac in physiological insulin secretion? Our own experimental data appear to provide some answers to this question. For example, using

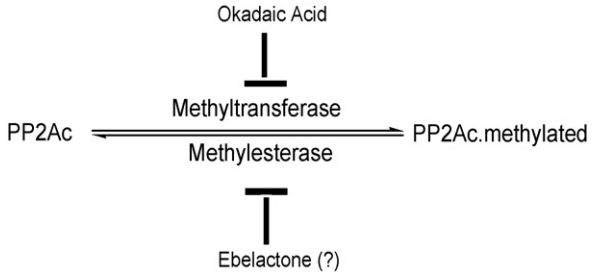

Fig. 3 – Post-translational carboxyl methylation–demethylation of PP2Ac: as described in the text, PP2Ac undergoes carboxylmethylation–demethylation cycle at its C-terminal leucine. Both the methylating and demethylating enzymes have been localized in the islet β cell [23,29]. Okadaic acid has been shown to inhibit the CML of PP2Ac in a structure-specific manner. Ebelactone, a more global inhibitor of esterases, has been reported to delay the demethylation of PP2Ac [see text for additional details]. However, as indicated in the text, it has also been shown to elicit inhibitory effects on several other enzymes, and therefore is indicated by [?] to represent potential limitations in its use.

insulin-secreting INS cells, we reported marked inhibitory effects of glucose [in intact cells] or its metabolites, such as citrate and phosphoenolpyravate [in cell lysates] on the CML of PP2Ac. In addition, mannoheptulose, an inhibitor of glucose metabolism, completely prevented the inhibitory effects of glucose on the CML of PP2Ac [26,27]. Based on these data, we proposed that GSIS may, in part, be due to the ability of glucose to inhibit specific protein phosphatases [e.g., PP2A] thereby retaining putative exocytotic proteins in their phosphorylated [active] conformation [27].

We next asked if the CML of PP2Ac exerts any effects on the enzymatic activity of PP2A since the existing experimental evidence provides conflicting conclusions with regard to potential contributory roles for the CML of PP2Ac on its phosphatase function. For example, using purified PP2Ac and carboxylmethyl transferase preparations, Favre et al. have demonstrated a 30–50% increase in the PP2A activity following the CML of PP2Ac [22]. Compatible with these data, we also observed a 25% increase in PP2A activity in INS cell and normal rat islet cytosolic fractions following methylation of the carboxy-terminal leucine of PP2Ac [23]. Interestingly, in contrast to these stimulatory effects, De Baere et al. observed no effects of the CML on catalytic function of either the dimeric or trimeric forms of PP2A [28]. This discrepancy might be due to (but are not limited to), differences in cell types and experimental conditions employed, including substrates used in PP2A activity measurements, and purified versus crude preparations of phosphatase and carboxylmethyl transferases utilized in these studies [29].

Lastly, we also investigated the relative half-life of the methylated PP2Ac to further understand if the methylation of PP2Ac is acutely regulated. We observed that the methylated PP2Ac underwent rapid demethylation [$t_{1/2}$ = 40 min] catalyzed by a methyl esterase localized in islet homogenates. Ebelactone, a known inhibitor of methyl esterases [23] (Fig. 3), significantly delayed [>200 min] the demethylation of PP2Ac. Further, it reversibly inhibited glucose- and ketoisocaproate-induced insulin secretion from normal rat islets. Together, our findings identified, for the first time, a methylation–demethylation cycle for PP2Ac in the β cell and suggest a key functional relationship between PP2A activity and the CML of PP2Ac. They also suggested novel roles for the CML of PP2Ac in nutrient-induced insulin exocytosis. In this context, it may be important to note that while okadaic acid specifically inhibits the CML of PP2Ac by binding to its the C-terminal region, and thus preventing its methylation, the specificity of ebelactone to inhibit PP2Ac methylesterase, however, remains to verified. Ebelactone has also been shown to modulate other enzymes including prenyl cysteine methyl esterases [30], cathepsin A [31], urinary kinases [32], carboxypeptidase Y-like kininases [33] and lipases [34,35]. Future studies will need to develop specific inhibitors for PP2Ac demethylases which can be employed to further decipher roles for these signaling steps in physiological insulin secretion. In conclusion, while the existing evidence appears to indicate biochemical [see above] and immunological [A. Kowluru, unpublished data] evidence in support of localization of PP2Ac methylating and demethylating enzymes in the islet β cell, the relative contributory roles for the CML of PP2Ac in the cascade of events leading to GSIS remain to be verified further [29].

2.3. *Glucose-induced methylation of protein arginines in insulin-secreting cells*

In addition to C-terminal cysteines and leucines, glucose-stimulated methylation of arginine has also been reported in insulin-secreting cells [36]. Using labeled SAM as the methyl donor, Lim et al. reported enzymatic methylation of a 20 kDa protein in clonal HIT cell preparations. They also reported a significant correlation between stimulation of the methylation of the 20 kDa protein [3.2-fold] and insulin secretion [2.5-fold] by glucose. Forskolin also increased the methylation of this protein significantly [nearly 4.5-fold]. Furthermore, the removal of extracellular calcium markedly inhibited the ability of glucose to stimulate the methylation of this protein and insulin secretion. Additional studies identified the methylated amino acid as arginine. Based on these and other findings, these investigators proposed that methylation of the endogenous 20-kDa protein might play regulatory role in insulin secretion [36]. Further identity of this 20 kDa and its regulatory role[s] in physiologic insulin release remain to be verified.

3. Studies on phospholipid methylation in pancreatic β cells

3.1. *Studies in intact β cells*

Enzymatic methylation of phospholipids has been a subject of extensive research during the past two–three decades. The most studied in the area of lipid methylation is the three successive methylation steps of phosphatidylethanolamine resulting in the formation of phosphatidylcholine, which is felt to increase membrane fluidity, but this topic continues to

be a matter of a significant debate [37–39]. While several studies have appeared in the 1980s to demonstrate glucose-dependent modulation of phospholipid methylation in islet β cells [see below], very little is known yet with regard to potential contributory roles of these signaling steps in GSIS either directly or indirectly. I will overview below the existing body of evidence on phosphatidylethanolamine methylation in the pancreatic β cell.

Saceda et al. [40] first reported glucose-mediated, transient stimulation of phospholipid methylation in rat pancreatic islets. They also provided the first evidence to suggest that a mixture of DL-homocysteine and 3-deazaadenosine inhibited glucose-induced phospholipid methylation. Based on these data, these investigators concluded that phospholipid methylation may regulate GSIS. At the same time, independent studies by Best et al. have provided additional support [41] for a modulatory role for phospholipid methylation in insulin secretion. They reported that the combination of 3-deazaadenosine and DL-homocysteine impaired the incorporation of ^{3}H-methyl group from L-[methyl-^{3}H]methionine into endogenous islet proteins and phospholipids, but failed to affect turnover in the phosphatidylinositol cycle. Further, the inhibitors of methylation decreased insulin release evoked by D-glucose or by variety of stimuli, including amino acids. They also observed that the inhibitors of methylation did not impair either the oxidation of D-glucose or affect its capacity to decrease K^{+} conductance, stimulate Ca^{2+} inflow and provoke ^{45}Ca accumulation in pancreatic islets. Based on these experimental findings, it was proposed that, in the process of insulin secretion, a methyl acceptor protein and/or phospholipid play(s) a limited modulatory role in the coupling of cytosolic calcium accumulation to insulin exocytosis.

Independent studies by our research group [42] have provided additional support for glucose-induced phospholipid methylation in the pancreatic islet. Using normal rat islets we reported a two-fold stimulation in the incorporation of [^{3}H-methyl] groups from [^{3}H-methyl] methionine into phospholipids within 6 min of exposure to a stimulatory glucose concentration. We also demonstrated that non-stimulatory sugars, L-glucose and D-galactose, failed to affect phospholipid methylation in islet cells. We have also provided evidence to indicate a significant degree of inhibition of phospholipid methylation in these cells following blockade of calcium channel activation by verapamil. These findings led us to conclude that phosphatidyl-ethanolamine methylation might play important regulatory roles in GSIS.

Studies from Laychock's laboratory [43] in intact islets have also demonstrated a significant stimulation of phospholipid methylation by stimulatory concentrations of glucose. Isoproterenol also enhanced lipid methylation and insulin secretion to a similar magnitude seen in the presence of glucose. These investigators reported no significant effects of 2-deoxyglucose, tolbutamide, nor 8-bromo-cyclic AMP on islet phospholipid methylation. Moreover, 3-deazaadenosine, a known generic inhibitor of methyl transferases, inhibited both glucose and isoproterenol-stimulated methyltransferase activity and insulin release. Based on these findings it was concluded that methylation of phospholipids plays a role in amplification of the β cell stimulus-secretion coupling response to specific secretagogues.

3.2. *Studies in broken cell preparations*

At least two previous investigations attempted to understand biochemical and functional properties of phospholipid methylating enzymes in insulin-secreting cells. First, studies from Laychock's laboratory [43] have demonstrated that the islet phosphatidylethanolamine-methylating enzymes were sensitive to and inhibited by S-adenosyl homocysteine, sodium deoxycholate and Triton X-100 in islet homogenates. Furthermore, provision of exogenous mono- or dimethyl phosphatidylethanolamine species increased the incorporation of [^{3}H]methyl groups into their cognate lipid species, further substantiating localization of individual methylating enzymes in the β cell. Isoproterenol, but not glucose, stimulated phospholipid methylation in islet homogenates. Second, subsequent studies by Kowluru et al. [44] have reported localization of a magnesium-requiring phospholipid methyl transferase activity in rat pancreatic islet lysates. Such an activity was doubled by calcium whereas cyclic nucleotides [e.g., cAMP or cyclic GMP] exerted no significant effects on this enzyme activity. Calcium increased the V_{max} of the enzyme without affecting its K_m with respect to SAM. Further, calmodulin antagonists, such as chlorpromazine, trifluoperazine, and dibucaine inhibited the calcium-stimulatable activity without affecting the activity in the absence of calcium. Phosphatidylserine stimulated, and arachidonic acid and palmitic acid inhibited, the basal enzyme activity. The methylated products were found to be primarily mono- and dimethylphosphatidylethanolamine [30%] and phosphatidylcholine [43%] and an, as yet unidentified, non-polar lipid fraction [27%], as judged by thin-layer chromatography. In the presence of calcium, incorporation of methyl groups into PE, mono- and dimethylphosphatidy-lethanolamine and non-polar lipids was increased by 131, 60, and 46%, respectively. Subcellular fractionation studies have suggested a nearly five-fold enrichment of this activity in insulin-containing secretory granules compared to other fractions [e.g., nucleus, mitochondria, microsomes or cytosol [44]]. Together, the above finding implicate a regulatory role for phospholipid methylation in GSIS from the pancreatic β cell.

Extant data also implicate key regulatory roles for phospholipid methylation in hormone secretion in multiple cell types. For example, studies by Hook et al. have reported a role for phospholipid methylation in corticotropin-releasing factor-induced corticotropin secretion in mouse pituitary tumor cells [45]. Along similar lines, Prasad and Edwards have documented a regulatory role for phospholipid methylation in thyrotropin-mediated secretion of thyroid hormone [46]. Prasad et al. have also provided evidence for the involvement of phospholipid methylation in neuropeptidergic stimulation of pituitary hormone secretion [47]. Clearly, these findings implicate roles for lipid methylation in receptor-mediated activation of hormone secretion, presumably coupled to the activation of G-protein-coupled receptors. In the case of the pancreatic β cell, however, as we discussed above, one or more metabolites of glucose and/or alterations in ionic balance appear to trigger phospholipid methylation transiently under conditions of stimulated insulin secretion. It is also important to note that non-metabolizable sugars elicited no effects on either the phospholipid methylation or

the CML of specific G-proteins, further substantiating the view point that glucose metabolic events may lead to activation of these pathways culminating in insulin secretion [see below].

Taken together, the above findings from multiple laboratories have provided convincing body of evidence in support of the formulation that phospholipid methylation might play a significant regulatory role in the stimulus-secretion coupling of GSIS. Despite such a compelling evidence, no follow-up studies have been carried out to further document if such an alteration[s] in glucose-induced phospholipid methylation would promote alterations in plasma membrane [e.g., fluidity and/or microviscosity], which may be conducive for granule docking and fusion for exocytotic secretion of insulin.

4. A model for a potential cross-talk between protein CML and phospholipid methylation in GSIS

Based on our current knowledge of the regulatory effects of glucose on protein and lipid methylation [as reviewed above], I propose a model for a potential cross-talk between these two pathways in signaling events leading to GSIS (Fig. 4). The generation of soluble second messengers and/or alterations in intracellular concentrations of ions [e.g., calcium], as a consequence of glucose metabolism, leads to activation of protein as well as phospholipid methylation. Several lines of evidence in multiple cell types, including the isolated β cell suggest that an increase in the CML of prenylated proteins [e.g., Cdc42, Rac1, Rap1, etc.] leads to an increase in their hydrophobicity. This, in turn, facilitates association of these activated proteins with their respective membranous compartments [e.g., plasma membrane or insulin-containing secretory granules]. This signaling step is critical for optimal interaction of activated G-proteins with their effector proteins, including p21-activated kinase and phospholipase C, etc.; these signaling steps appear to be requisite for GSIS [[48] and unpublished data from our laboratory].

Although not verified in β cells thus far, it is likely that glucose-mediated activation of phospholipid methylation, and subsequent generation of phospholipids [e.g., phosphatidylcholine] could result in alterations in membrane properties [e.g., microviscosity and fluidity], thereby facilitating optimal docking and fusion of secretory granules with the plasma membrane. This needs to be verified. Furthermore, we have demonstrated that generation of biologically active lipids [e.g., phosphatidylcholine] promote dissociation of inhibitory factors/proteins [GDP-dissociation inhibitor; [49]] from specific G-proteins [e.g., Rac1], to pave way for their activation [50]. It is also important to note that earlier studies from our laboratory have demonstrated localization of the phospholipid methyl transferase in the secretory granule fraction, and its regulation by calcium raises as interesting possibility for the direct involvement of this signaling step in insulin exocytosis. Additional studies are needed to verify this formulation [44].

Interestingly, using isolated rat liver plasma membranes, Hashizume et al. [51] have reported dual regulatory roles for GTP on phospholipid methylating enzymes. They reported a significant inhibition by GTP of methyltransferase 1, which mediates the methylation of phosphatidyl-ethanolamine to phosphatidyl-*N*-mono-methylethanolamine. In contrast, methyltransferase II, which catalyzes the formation of phosphatidyl-*N*,*N*-dimethylethanolamine or phosphatidylcholine, was markedly stimulated by GTP. Based on these observations, it was proposed that GTP plays regulatory roles in phospholipid methylation in the liver plasma membrane. These data raise an interesting possibility of potential regulation of phospholipid methyl transferase by G-proteins, which again remains to be determined.

The next important question is what is the role, if any, of the CML of PP2Ac [and its activation] in glucose-induced activation of proteins or lipid methylation events leading to insulin secretion? I propose that the activation of PP2A does represent

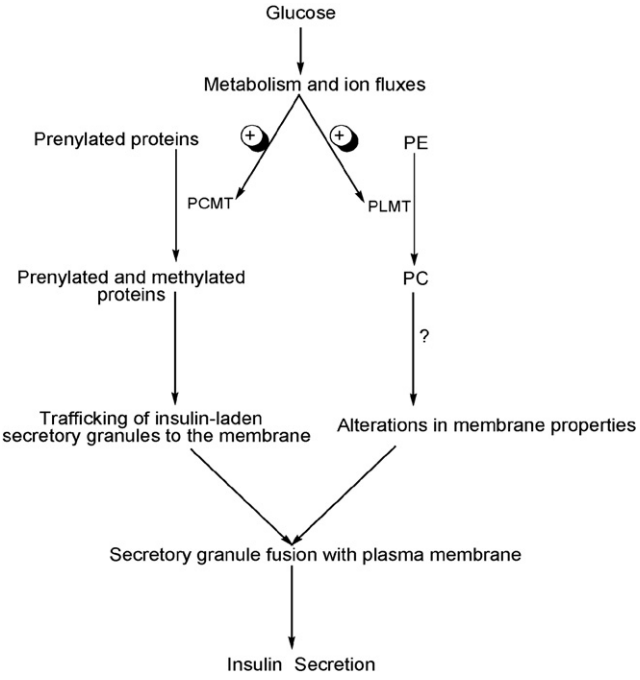

Fig. 4 – Our proposed model for potential involvement of protein and lipid methylation steps in GSIS: glucose metabolism leads to the generation of soluble second messengers and/or alterations in intracellular concentrations of ions [e.g., calcium]. This, in turn, leads to activation of G-protein CML and PL methylation. Increase in the CML of prenylated proteins [e.g., Cdc42, Rac1, Rap1, etc.] leads to an increase in their hydrophobicity, culminating in their association with their respective membranous sites [e.g., plasma membrane or insulin-containing secretory granules]. Further, increase in PE methylation leads to generation of PC, which promotes dissociation of Rac1 from GDI-Rac1 complex, to pave way for its activation. It is also likely that PL methylation promotes alterations in the membrane, which might favor fusion of insulin-containing secretory granules with the plasma membrane followed by exocytotic secretion of insulin. Please see text for potential roles for CML of PP2Ac in bridging the two signaling pathways leading to GSIS. PCMT, Protein carboxylmethyltransferase; PLMT, phospholipid methyltransferase; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

an important step in connecting these signaling pathway. First, it has been postulated, and even established in certain cell types [see ref. [29] for a review] that the CML of PP2Ac promotes its association with other subunits [e.g., the scaffolding and regulatory subunits] of the holoenzyme, and subsequently its catalytic activity. Recent findings of Wang et al. [48] have demonstrated glucose-induced phosphorylation and activation of p21-activated kinase, which is an effector protein for small G-proteins, such as Cdc42 and Rac1, which have been implicated in GSIS. It has also been shown in other cell types that specific regulatory proteins of small G-protein [e.g., Rac1] function and activation [e.g., GDP-dissociation inhibitor] are controlled by phosphorylation-dephosphorylation reactions [52]. We have demonstrated recently that GDP-dissociation inhibitor plays a negative modulatory role in GSIS [50]. Albeit controversial, it has also been demonstrated that specific enzymes of phospholipid methylation cascade are regulated by phosphorylation/dephosphorylation [53–55]. This topic remains still unexplored in the islet β cell.

5. Alterations in lipid and protein methylation in various pathological states

Another important area of investigation is to examine the functional status of the G-proteins and/or phospholipid methylation pathways in islet β cells derived from models of impaired insulin secretion. Indeed, studies from several laboratories have reported alterations in phospholipid methylation in various cell types derived from animal models of diabetes, but not the pancreatic islet. For example, Ganguly et al. [56] have demonstrated a significant increase in phospholipid methylation in diabetic myocardium, which was reversed by insulin treatment. Taira et al. reported a marked increase in the phospholipid methylation in skeletal muscle of diabetic rats [57]. Kowluru and Kowluru have reported [58] significant alterations in relative abundance of methylated lipids in erythrocytes from streptozotocin diabetic rats. More recent studies by Hartz et al. have reported a marked increase in the expression of phospholipid methyl transferase in hepatic cells derived from the streptozotocin diabetic animals. Insulin treatment of the diabetic animals prevented the diabetes-induced increase in phosphatidyl-ethanolamine methyltransferase activity and abundance [59]. Together, the above described evidence appears to suggest potential alterations in the phospholipid methylation in the diabetic sate. It also appears that phospholipid methyl transferase activities and the CML of specific proteins [e.g., PP2Ac] are altered in other pathological states as well. For example, Guan et al. reported a decrease in the activity of phospholipid methyl transferase activity in the frontal cortex of brain in patients with Alzheimer's disease [60]. Along these lines, Sontag et al. [61] have recently suggested that down-regulation of the CML of PP2Ac and methyltransferase activity may contribute toward the pathogenesis of Alzheimer's disease. Potential alterations in the carboxylmethylation–demethylation status of G-proteins in pathological states such as diabetes or Alzheimer's remain to be investigated. For the above stated reasons, it may be necessary to investigate potential alterations, if any, in the ability of glucose to precisely regulate the methylation of C-terminal cysteines [e.g., G-proteins] and leucines [e.g., PP2Ac] as well as phospholipids [e.g., phosphatidylethanolamine] in β cells derived from models of impaired insulin secretion [e.g., islets from animal models of diabetes or islet β cells chronically exposed to high glucose and/or high lipid environment]. Data from such an investigation[s] are critical to determine if the abnormalities in insulin secretion in these model systems are, in part, due to defects in these signaling pathways. If so, it should then be possible to develop novel therapeutic interventional modalities to prevent and/or restore these signaling defects to achieve normal islet β cell function.

6. A need for the development of novel pharmacological inhibitors for protein and lipid methylation

While adequate evidence is already available on regulation of protein and lipid methylation by glucose in islet β cells, very little is known with regard to potential inter-dependence of each of these pathways in the cascade of events leading to insulin secretion (Fig. 4). One plausible approach is to quantitate glucose-induced effects on the CML of specific proteins [e.g., Cdc42 or Rac1] in isolated β cells in which phospholipid methyltransferases are compromised either via pharmacological or by molecular biological manipulations. With regard to pharmacological inhibitors of phospholipid methylation, extant studies have utilized classical methylation inhibitors, including *S*-adenosyl homocysteine, 3-deazaadenosine, 3-deazaaristeromycin, etc. [62]. Bezafibrate, clofibric acids and calcium channel blockers [e.g., verapamil] have also been shown to inhibit phospholipid methylation [42,63,64], but again, they might exert untoward non-specific effects. Studies by Seo et al. [65] reported localization of an endogenous proteinacious inhibitor in porcine liver preparations with a significant inhibitory properties against several classes of SAM-dependent methylases, including protein and lipid methylases. Additional studies from this laboratory have identified this inhibitor to be as an oligosaccharide-linked acyl carrier protein [65]. Kido et al. [66] reported novel inhibitory properties of methylthiodeoxyadenosine [MTA] and its analogs against phospholipid methylation. Using an in vitro assay for invasion of rat ascites hepatoma cells, these researchers have demonstrated that MTA and five of its analogs [e.g., difluoro-MTA, deoxyadenosine, sinefungin, phenylthiodeoxyadenosine, fluorophenylthiodeoxyadenosine] specifically inhibited tumor cell invasion without significantly affecting cell proliferation. Further, they reported significant inhibition by these compounds of phosphatidylethanolamine methylation, but not protein CML. However, no follow-up studies have been carried out using these inhibitors. Therefore, due to a relative paucity in the availability of specific inhibitors, development of a more specific inhibitors of phospholipid methyl transferases does indeed represent a novel area of investigation. In contrast to the inhibitors of phospholipid methylation, relatively more specific inhibitors of protein CML are available, and have been utilized to decipher regulatory roles for this signaling step in GSIS. More recent studies by Baron et al. [67] have described the development of a novel class of indole-based small molecules as inhibitors for isoprenylcysteine carboxyl methylatransferases. Therefore, it

may be possible to verify if the signaling step of glucose-induced CML of specific proteins underlies either upstream or downstream to phospholipid methylation. While this appears to be a testable model, we are still faced with a difficult task of identifying specific pharmacological inhibitors for phospholipid methylation signaling steps. For these reasons, I feel that there is an immediate need for the development such pharmacological probes to better understand the regulatory roles for lipid and protein methylation steps in the cascade of events leading to hormone secretion.

7. Conclusions

I hope that this commentary provided a detailed review the existing evidence on the regulation, by glucose and other insulin secretagogues, of post-translational CML of specific proteins in the β cell, including the small molecular weight G-proteins, the γ subunits of trimeric G-proteins and the catalytic subunit of PP2A. I also made an attempt to summarize the experimental evidence, which implicates regulation, by glucose and other insulin secretagogues, of phosphatidylethanolamine methylation in the islet β cell. Based on these discussions, I proposed a working model to determine potential cross-talk, if any, between the protein and lipid methylation pathways in the regulation of GSIS. Existing literature also indicates significant abnormalities in these signaling pathways in pathophysiological states, including diabetes and Alzheimer's disease. Future studies will need to determine if the functional status of these pathways is affected in islets derived from [animal or human] models of impaired insulin secretion. It is also emphasized that there is an immediate need to identify and/or develop specific pharmacological inhibitors which can be employed to further decipher roles for these methylation reactions in islet β cell function.

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